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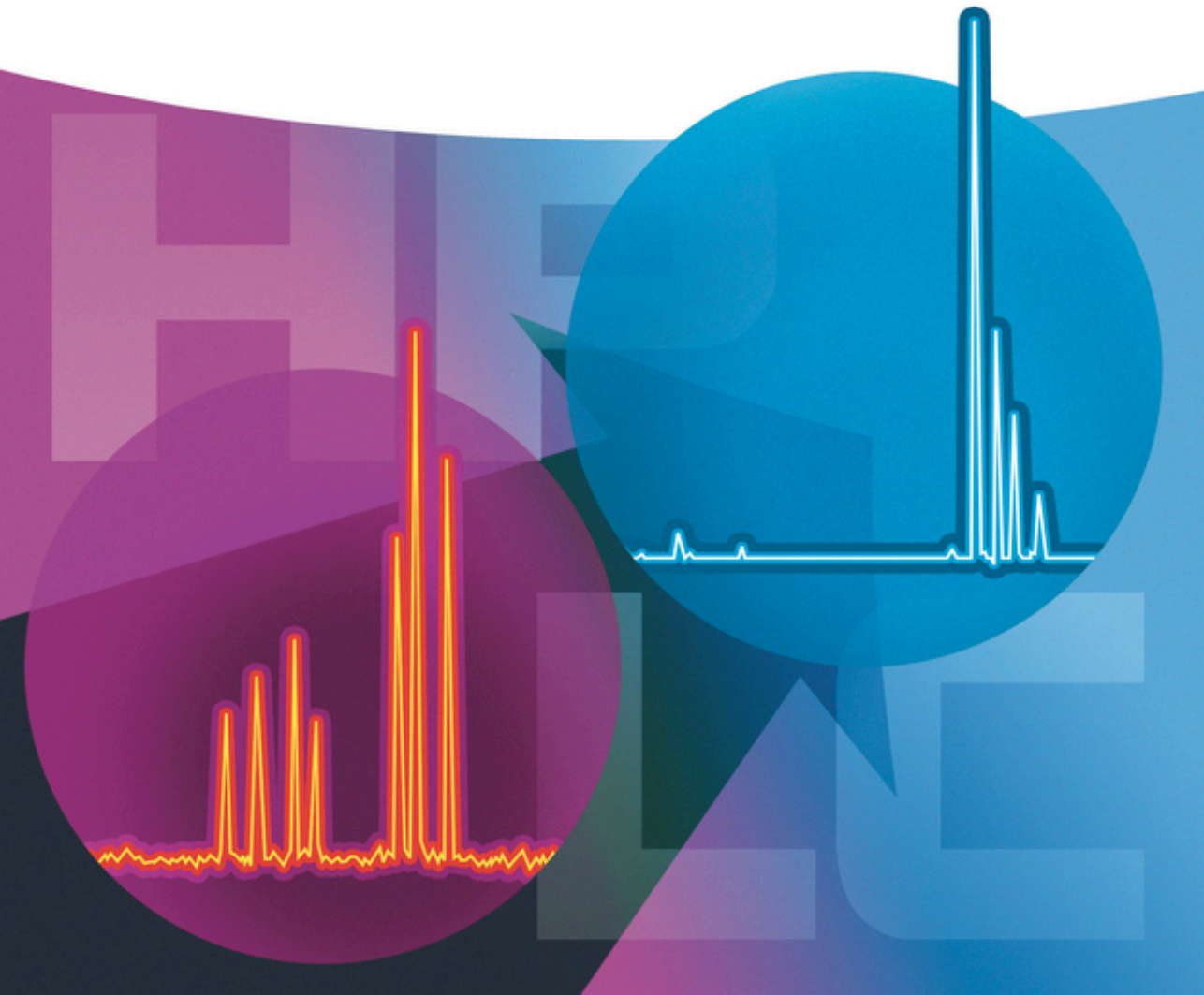
Edited by Stavros Kromidas

Optimization in HPLC

Concepts and Strategies



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Contents

Preface

The "HPLC world" is a diverse one – a lucky chance and challenging at the same time. Successful strategies for a "good" result can therefore look completely different.

The aim of this book is to provide interested colleagues successful strategies and proven ways for method development and optimization for all important areas in the field of HPLC and UHPLC. With this goal in mind, experts were invited to present their knowledge and experience in a practical and compact manner.

It was important to take both into account: Different challenges of a chromatographic nature, but also different framework conditions in everyday life. Only this enables a differentiated perspective and consequently a target-oriented approach: Hence, the authors are researchers or employees of well-known manufacturers, are service providers in industrial companies or private laboratories, or they have developed tools themselves.

Readers may find inspiration in the book for developing their individual optimization strategy.

I would like to thank my fellow authors for their time and commitment as well as WILEY-VCH, who made the realization of this project possible.

Blieskastel, June 2021 *Stavros Kromidas*

About the Book

The book is designed as a guide and does not have to be read in a linear fashion. The individual chapters represent self-contained modules; it is possible to "jump" at any time. In this way, we have tried to do justice to the book's character as a reference and hope that readers may benefit from this.

The book consists of four parts:

Part I: Optimization Strategies for Individual Problems

In the first part, optimization strategies for different analytes are discussed, from small molecules and chiral substances to biomolecules. Different modes of operation are also covered: LC–MS, 2D-HPLC, HILIC, SFC. Finally, optimization strategies based on structural info of the analytes are presented, and optimization possibilities in a regulated environment are discussed.

Part II: Computer-Aided Strategies (In silico Applications)

In Part II, concepts for computational method development for small molecules and biomolecules are presented, based on specific problems.

Part III: Users' Report

Service providers from two industrial companies and two private laboratories present their concepts for method development in Part III, based on the specifications and requests of internal and/or external customers.

Part IV: Manufacturers' Report

Employees of 5 well-known HPLC manufacturers show how the design of HPLC instruments, different tools, and the underlying philosophy support HPLC users in establishing the most efficient HPLC method possible, adapted to the problem at hand.

Part I

Optimization Strategies for Different Modes and Uses of HPLC

1.1

2D-HPLC – Method Development for Successful Separations

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1.1.1 Motivations for Two-Dimensional Separation

Historically, much of the research devoted to multidimensional separations and their application to real analytical problems has been focused on dealing with complex samples. These have traditionally been described as containing hundreds or thousands of compounds and are often derived from natural sources such as plant extracts or body fluids (e.g. blood or urine). Increasingly, however, we observe that multidimensional separation can be exquisitely effective for dealing with samples containing analytes that are difficult to separate but are not complex by the traditional definition. Since this distinction can have a big impact on how one approaches method development, we start here by explicitly differentiating the two cases.

1.1.1.1 Difficult-to-Separate Samples

The difficulty associated with a separating a particular sample may originate from its sheer complexity (i.e. thousands of compounds). In this case relying on chromatographic separation alone will not be enough to fully separate the mixture, and some other source of selectivity will be needed (e.g. sample preparation, and/or selective detection such as mass spectrometry). However, it is now common to encounter samples that contain only a few compounds but are difficult to separate simply due to the high degree of similarity of the compounds in the mixture. For example, a mixture may only contain six compounds, but if two of those six compounds are enantiomers (**1a** and **1b**), then fully separating the mixture using a single column may be difficult even if the separation of compounds 2–5 from **1a**/**1b** is straightforward. Such situations are encountered more frequently now compared to the past, in part due to the development of small-molecule drugs with multiple chiral centers [1], and the increasing recognition of the importance of both the D- and L- enantiomers of amino acids ([2], see Chapter 1.7), for example.

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1.1.1.2 Complex Samples

As stated above, traditionally complex samples have been thought of as containing hundreds or thousands of different compounds. These samples often come from nature, but not always. For example, surfactants and polymers produced by chemical synthesis can result in highly heterogeneous mixtures of thousands of different compounds. Historically, the analysis of such samples by multidimensional chromatography has been mainly focused on so-called comprehensive methods of separation that yield a kind of global profile or "fingerprint" of the contents of the sample. However, in cases where only one or a few particular molecules in the sample are of importance to the analysis, simpler multidimensional separation methods such as heartcutting can be adequate, and even preferred.

1.1.1.3 Separation Goals

As is often discussed in the multidimensional separation literature, and below, the process of developing a multidimensional separation method is one full of compromises. For example, conditions that favor shorter analysis times do not lead to the best detection sensitivity, and vice versa. Therefore, it is important for the analyst to identify – at the very beginning of method development – what are the characteristics or performance metrics for the method that are most important to him/her. For example, if achieving baseline resolution of six critical pairs of analytes is critically important for the method to be successfully applied, then method development decisions should support this objective, even if it comes at the cost of increased analysis time, and/or lower detection sensitivity.

1.1.2 Choosing a Two-Dimensional Separation Mode

All two-dimensional separations can be executed either "offline" or "online." In the offline mode, one or more fractions of ^{1}D effluent are collected in some kind of storage device such as a set of vials or a wellplate. These fractions are then injected at some later time (minutes to years) into another LC system (i.e. the same LC system running different conditions from the ^{1}D separation, or a different LC system altogether), either with or without intermediate processing of these fractions. For example, in proteomics applications of 2D-LC, it is common to desalt the fractions, or dry them down by evaporation to remove organic solvent, before analysis by the ^{2}D separation [3]. In the online mode, fractions collected from the ^{1}D column are either processed immediately by direct injection into the ^{2}D column, or stored for a short time (seconds to hours) in some kind of device (typically capillary loops or sorbent-based traps) that is internal to the instrument. An example of an instrument configuration commonly used for this purpose is shown in Figure 1.1.1. In this case, the interface valve situated between the ^{1}D and ^{2}D columns has two positions. Switching between them changes the roles of loops 1 and 2 between collecting ^{1}D effluent and introducing the fraction of the ^{1}D effluent into the ^{2}D flow stream, effectively injecting that material into the ^{2}D column.

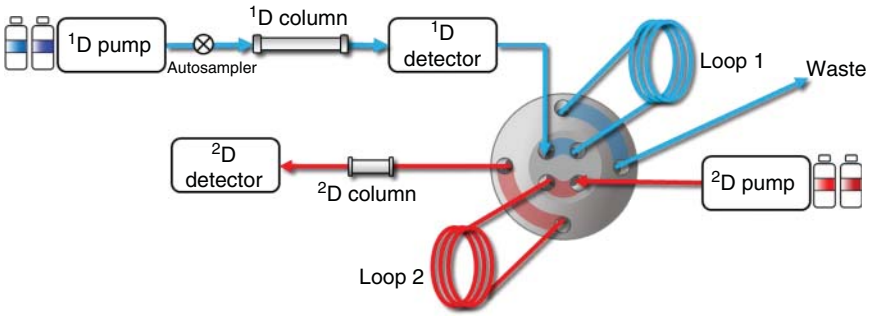

Figure 1.1.1 Illustration of an instrument configuration typically used for 2D-LC. *Source*: Dr. Gabriel Leme.

As commercially available equipment for 2D-LC separation has become more sophisticated and reliable, the trend in the industry has been to move away from offline separations because of challenges associated with implementation of offline separations for large numbers of samples, and with degradation and contamination of ^{1}D effluent fractions when they are handled external to the instrument [4]. Given this trend, I have chosen to focus entirely on online 2D-LC for the rest of this chapter. Readers interested in learning more about offline 2D-LC are referred to review articles dedicated to this topic [5, 6].

1.1.2.1 Analytical Goals Dictate Choice of Mode

Starting in the late 1970s, different groups began developing the modes of 2D-LC separation we have come to know as "heartcutting" and "comprehensive" [4, 7]. In the most recent decade, two additional modes have been developed, which are now known as "multiple heartcutting" and "selective comprehensive" 2D separations. Each of these four modes will be discussed in some detail in Section 1.1.2.2. At this point, though, I want to emphasize that choosing which separation mode you will use should be driven by the overall goals of the analysis. For example, if you have a complex sample and you want to learn as much as you can about that sample (i.e. identify hundreds of compounds), then the comprehensive mode of 2D separation will almost always be the best choice. However, if you are only interested in a few target compounds in the sample – even if the sample matrix is highly complex – then a more targeted mode of 2D separation such as heartcutting or multiple heartcutting will likely be the best approach. In practice, time spent on each ^{2}D separation is one of the most precious resources of the 2D-LC instrument, and allocating effort to ^{2}D separations that are not necessary to achieve the overall analytical goals of the analysis is costly (in terms of both time and supplies), wasteful, and adds unnecessary complexity to the method.

1.1.2.2 Survey of Four 2D Separation Modes

The vast majority of 2D-LC applications being developed today fit into one of the four modes of 2D separation illustrated in Figure 1.1.2. In the single-heartcut mode

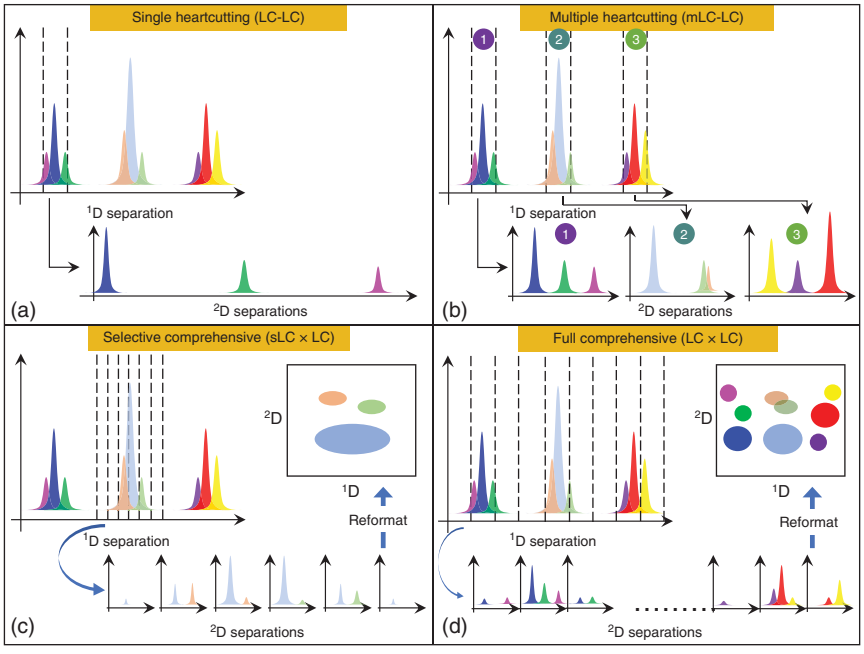

Figure 1.1.2 Illustration of four different modes of 2D-LC separation.

(A; LC–LC), a single fraction of ^{1}D effluent containing analytes of interest is captured at the outlet of the ^{1}D column and transferred to the ^{2}D column where this submixture of the original sample will be further separated if the separation mechanisms employed in the first and second dimensions are complementary. Perhaps the biggest advantage of the LC–LC mode is that the time that can be dedicated to separation of the ^{1}D effluent fraction in the second dimension is not strictly limited. This provides tremendous flexibility in terms of choosing parameters for the ^{2}D separation, including flow rate, column dimensions, and injection volume. The biggest disadvantage of LC–LC, however, is that the scope of the analysis is limited. We are restricted to the analysis of compounds that can be captured in a single fraction of ^{1}D effluent. Nevertheless, the LC–LC approach has been used to great effect in application areas ranging from identification of small-molecule pharmaceutical impurities [8] to the detection of drug metabolites in plasma [9].

The extreme opposite of LC–LC in terms of analytical scope is the comprehensive mode of 2D separation (D; LC × LC). As the illustration shows, in this case, fractions of ^{1}D effluent are collected and transferred – one at a time, in a regular, serial fashion – to the ^{2}D separation. Typically, this results in a long string of many (tens to hundreds) ^{2}D chromatograms collected in a single detector datafile. This long data string can then be parsed into pieces that correspond to individual ^{2}D separations and reformatted to produce a two-dimensional data array, which can then be viewed either as a contour map or a 3D surface rendering of the data. The advantages and disadvantages of the LC × LC approach are effectively the converse of those for the LC–LC approach. The main advantage is that the scope of the ^{2}D

separation is as wide as the scope of the ^{1}D separation; the main disadvantage is that the time that can be dedicated to each ^{2}D separation is severely restricted because of the sheer number of fractions of ^{1}D effluent that must processed by the second dimension.

The two other modes illustrated in Figure 1.1.2 are hybrids of the LC–LC and LC × LC modes. In the case of multiple heartcutting (B; mLC–LC), one fraction of ^{1}D effluent is collected per region of the ^{1}D separation targeted for further separation, just like LC–LC, but this is repeated two or more times over the course of the 2D separation. Finally, in selective comprehensive separations (C; sLC × LC), multiple fractions of ^{1}D effluent are collected across a zone of interest in the ^{1}D separation, stored in loops or traps associated with the interface, and then injected one at a time into the ^{2}D column as in LC × LC separations. These hybrid modes are attractive in many situations because they capitalize on the strengths of LC–LC and LC × LC while mitigating their weaknesses. Specifically, mLC–LC and sLC × LC provide the analyst with a lot of flexibility in development and implementation of a 2D-LC method because they provide a means to decouple the process of collecting ^{1}D effluent fractions from the process of further separating those fractions in the second dimension [10].

1.1.2.3 Hybrid Modes Provide Flexibility

There are multiple ways that the added flexibility provided by mLC–LC and sLC × LC is practically useful, but I provide two examples for consideration here. First, sLC × LC is helpful for avoiding the so-called undersampling problem in 2D separations. Undersampling refers to the negative effect of collecting ^{1}D effluent fractions that are wider than about one-half of a ^{1}D peak width, whereby analytes eluting closely from the ^{1}D column are mixed back together in the sampling process. This effectively diminishes the performance of the first dimension of a 2D separation [11–13]. Overcoming this problem in the LC × LC mode is especially difficult when ^{1}D peaks are narrow (e.g. less than five seconds wide), but the sLC × LC mode can be used to manage this challenge by collecting several narrow (as low as one second or less) fractions over a particular region of interest in the ^{1}D separation. Second, sLC × LC can also be used to manage the volume of ^{1}D effluent that is injected into the ^{2}D column for each region of interest in the ^{1}D separation. A concrete example will make this benefit more clear. Suppose we have an existing 1D-LC separation running at 1 mL/min and we want to transfer a particular peak of interest to a ^{2}D column for further separation, and/or characterization by mass spectrometry. If the ^{1}D peak is 15 seconds wide, then the volume of the peak that has to be transferred is 250 µl. While it is certainly possible to transfer this volume in a single fraction, there are many cases where injecting such a large volume into the ^{2}D column will compromise the performance of the ^{2}D separation, especially when there is a mismatch between the mobile phases used in the ^{1}D and ^{2}D separations [14]. With sLC × LC, however, one could collect four fractions of the ^{1}D peak of interest instead of one, with each of the fractions being about 60 µl, and then these four fractions would be injected into the ^{2}D column one at a time [15]. This, of course,

is likely to add time to the overall analysis and requires a more complex interface, but this kind of flexibility can be very valuable during method development.

1.1.3 Choosing Separation Types/Mechanisms

Once one has chosen which mode of 2D separation to use, the next most important decision involves choosing which two separation types will be used in the first and second dimensions of the 2D system.

1.1.3.1 Complementarity as a Guiding Principle

There has been much discussion in the 2D separations literature about the principle of "orthogonality" as it is related to choosing two separation types to use in a 2D separation. The reason for invoking orthogonality is that – from a purely theoretical standpoint – it is best if the retention patterns obtained from the ^{1}D and ^{2}D separations are not at all correlated [16]. However, I think it may be more practically relevant to think about the relationship between two separation types used in the 2D separation in terms of complementarity. To what extent does the separation type used in the second dimension complement the separation already used in the first dimension? A concrete chemical example will help make this point. Suppose we are separating a mixture of peptides that vary in both the total number of amino acids and the number of lysine residues such that the degree of positive charge on these peptides in solution varies as well (low pH). If we set up a 2D-LC system with reversed-phase C18 columns and low pH mobile phases in both dimensions, this will not yield an effective 2D separation because the ^{2}D separation does not add anything new to the separation in terms of selectivity. On the other hand, suppose we change the ^{1}D separation to cation-exchange (CEX) where peptides will elute mainly according to their degree of positive charge (low charge elutes first, high charge elutes last). Now, if we add a ^{2}D separation using a reversed-phase (RP) C18 column, this will nicely complement the ^{1}D separation because it will separate mainly according to the water solubility of the peptides (most soluble elutes first, least soluble elutes last). In this case, we can have two peptides that carry the same charge – and thus coelute from the CEX separation – but have very different water solubilities due to differences in the number and/or type of amino acids, and can be easily separated by the ^{2}D RP column.

Historically, a lot of effort has been dedicated to learning which separation types are most complementary for different sample types and applications. New users can use this prior research as a foundation for their own work. For some application areas, there are specific papers that illustrate the complementarity of different separation types for specific types of molecules such as peptides [17]. I encourage readers to consult databases of 2D-LC applications to quickly learn about which two separation types might be useful for their application (http://www.multidlc.org/literature/2DLC-Applications).

1.1.3.2 Pirok Compatibility Table

Unfortunately, we need to consider more than just the complementarity of the selectivities of two separation types used in a 2D-LC separation. Other factors such as the compatibility of the mobile phases used with each separation type are often important, and in fact can render useless a pairing of separation types that looks quite attractive from the point of view of selectivity. For example, pairing a normal-phase (NP) separation (i.e. bare silica stationary phase; hexane mobile phase) with an RP separation is attractive for some applications because the NP separation is dominated by adsorptive analyte–stationary phase interactions, whereas the RP separation is dominated by the partitioning of analytes into a bonded stationary phase. This difference in retention mechanisms can lead to highly complementary selectivities. However, we encounter a major practical difficulty in this case because the nonpolar organic solvent–rich mobile phases used for NP separations are not miscible with the water-rich mobile phases used for RP separations – at least not across a wide range of compositions. This difficulty has limited the use of some combinations of separation types such as NP–RP, although even in this case the miscibility problem can be managed by injecting very small volumes of ^{1}D effluent into large ^{2}D columns [18]. Pirok and Schoenmakers have summarized a lot of the knowledge in the 2D-LC field about which separation types work well together using the table shown in Figure 1.1.3. Combinations shaded with green colors are likely to work well, whereas combinations shaded with red colors present at least one major difficulty that will have to be managed if they are chosen for a 2D separation. Readers interested in a more detailed explanation of all of the information in this table are referred to the original paper of Pirok and Schoenmakers [19]. The table is also being updated as 2D-LC technology evolves; a current version can be found at our website (www.multidlc.org/megatable).

1.1.3.3 Measuring the Complementarity of Separation Types

Once we have made initial decisions about which two separation types to use in our 2D-LC separation, we need to assess the quality of the resulting separations. For more targeted separations, usually we are most interested in resolving one or more target compounds from the sample matrix, or from themselves. In this case, it is a straightforward matter to evaluate the extent to which the ^{2}D separation has resolved compounds that coeluted from the ^{1}D column, and thus the complementarity of the two separation types. For more comprehensive separations, we are usually interested in the extent to which the ^{1}D and ^{2}D separations – working together – spread the constituents of the sample out across the entire separation space. The need to assess this has led many groups to develop a variety of metrics, which have been critically discussed and compared in recent articles [20, 21]. In our own work, we have used the approach illustrated in Figure 1.1.4, which amounts to estimating the fraction of the available 2D separation space that is occupied by peaks by counting the number of bins that are occupied by peaks and dividing by the total number of available bins in the space. During method development, we adjust elution conditions in both dimensions to spread the peaks out as much as possible with the goal of reaching

Figure 1.1.3 Matrix illustrating the compatibility of different separation modes when used in the first or second dimension of 2D-LC systems. *Source*: Reprinted with permission from ref. Pirok et al. [19]. Licensed under CCBY 4.0.